



Bierzynska, A., McCarthy, H., Soderquest, K., Sen, E., Colby, E., Ding, W., Nabhan, M., Kerecuk, L., Welsh, G., Saleem, M., Hedge, S., Hughes, D., Marks, S., Feather, S., Jones, C., Webb, N. J. A., Ognjanovic, M., Christian, M., Gilbert, R. D., ... Koziell, A. (2017). Genomic and clinical profiling of a national Nephrotic Syndrome cohort advocates a precision medicine approach to disease management. *Kidney International*, 91(4), 937–947.
<https://doi.org/10.1016/j.kint.2016.10.013>

Peer reviewed version

Link to published version (if available):
[10.1016/j.kint.2016.10.013](https://doi.org/10.1016/j.kint.2016.10.013)

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This is the accepted author manuscript (AAM). The final published version (version of record) is available online via Science Direct at <https://doi.org/10.1016/j.kint.2016.10.013> . Please refer to any applicable terms of use of the publisher.

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Genomic and clinical profiling of a national Nephrotic Syndrome cohort advocates a precision medicine approach to disease management

Agnieszka Bierzynska¹, Hugh J. McCarthy¹, Katrina Soderquest², Ethan S. Sen¹, Elizabeth Colby¹, Wen Y. Ding¹, Marwa M Nabhan³, Larissa Kerecuk⁴, Shivram Hegde⁵, David Hughes⁶, Stephen Marks⁷, Sally Feather⁸, Caroline Jones⁹, Nicholas J A Webb¹⁰, Milos Ognjanovic¹¹, Martin Christian¹², Rodney D Gilbert¹³, Manish D. Sinha¹⁴, Graham Lord², Michael Simpson⁴, Ania B. Koziell², Gavin I. Welsh¹, and Moin A. Saleem¹

¹Bristol Renal and Children's Renal Unit, School of Clinical Sciences, University of Bristol, United Kingdom

²Division of Transplantation Immunology and Mucosal Biology, Department of Experimental Immunobiology, Faculty of Life Sciences and Medicine, King's College London, London, UK

³Egyptian group for orphan renal diseases (EGORD), Department of paediatrics, Kasr Al Ainy School of Medicine, Cairo University, Egypt

⁴Birmingham Children's Hospital

⁵University Hospital of Wales, Cardiff

⁶Royal Hospital for Children, Glasgow

⁷Great Ormond Street Hospital, London

⁸St James's University Hospital, Leeds

⁹Alder Hey Children's Hospital, Liverpool

¹⁰Department of Paediatric Nephrology and NIHR/Wellcome Trust Clinical Research Facility, University of Manchester, Manchester Academic Health Science Centre, Royal Manchester Children's Hospital, Manchester, UK

¹¹Newcastle upon Tyne Hospitals NHS Foundation Trust

¹²Nottingham Children's Hospital at the QMC

¹³Southampton Children's Hospital and University of Southampton School of Medicine

¹⁴Guy's and St. Thomas' Hospital, London

¹⁵Division of Genetics and Molecular Medicine, Faculty of Life Sciences and Medicine, King's College London, London, UK

Words: 2910

Key Words: Cytoskeleton; Focal Segmental Glomerulosclerosis; nephrotic syndrome; paediatric nephrology; podocyte; proteinuria

Abstract

Steroid Resistant Nephrotic Syndrome (SRNS) in children and young adults has differing aetiologies with monogenic disease accounting for 2.9-30% in selected series.

We aimed, using whole exome sequencing, to stratify a national population of SRNS children into monogenic and non-monogenic forms, and further define those groups by detailed phenotypic analysis

Paediatric SRNS patients were collected via a national UK Renal Registry (RaDaR). Whole exome sequencing (WES) was performed on 187 patients, of which 12% had a positive family history. Analysis focused on the 53 genes currently known to be associated with NS. Genetic findings were correlated with individual case disease characteristics.

Disease causing variants were detected in 26.2% of patients. Most occurred in the three most commonly SRNS associated genes: *NPHS1*, *NPHS2* and *WT1* but also in 14 other genes. Genotype did not always correlate with expected phenotype, e.g. mutations in *OCRL*, *COL4A3* and *DGKE*, associated with specific syndromes, were detected in isolated renal disease. Analysis by primary/presumed vs. secondary steroid resistance revealed 30.8% monogenic disease in primary compared to 0% in secondary SRNS permitting further mechanistic stratification. Genetic SRNS progressed faster to end stage renal failure, with no documented disease recurrence post-transplantation within this cohort. Primary steroid resistance in which no gene mutation was identified had a 47.8% risk of recurrence

In this unbiased paediatric population, WES allowed screening of all current candidate genes. Together with deep phenotyping we propose that this is an effective tool for early identification of SRNS aetiology, yielding an evidence-based algorithm for clinical management.

Introduction

Nephrotic syndrome is a heterogeneous entity only recently divided into mechanistic categories. Although population analyses have been limited in paediatric cohorts¹⁻³, idiopathic nephrotic syndrome (INS) has an estimated incidence of around 2 - 5/100,000 children/year depending on ethnic background². INS is currently classified into Steroid Sensitive (SSNS), or Steroid Resistant (SRNS), with at least 2.9-30% of SRNS cases (in series variably enriched for consanguineous disease or other phenotypes) now known to have an underlying Mendelian, genetic cause. A challenging subset of cases, considered to be immunologically mediated and caused by an as yet unidentified circulating factor(s) can present as secondary steroid resistance after initial steroid sensitivity.⁴ The most dramatic evidence of the presence of a 'circulating factor' is rapid recurrence of nephrotic disease soon after kidney transplantation in 40-60% of graft recipients with SRNS.

Approximately 70% of children with idiopathic NS respond to steroids and other immunosuppression, whereas the remaining 30% are resistant to this therapy at the outset and have primary SRNS. Of the 70%, a further 20 to 30% of cases who initially have steroid sensitive disease become steroid resistant, termed secondary SRNS. Histological appearance has limited correlation to pathomechanism, but renal biopsy of SRNS generally shows focal segmental glomerulosclerosis (FSGS). There are currently no robust or reliable clinical indicators or biomarkers of response meaning that prediction of disease progression or response to medication cannot be clearly defined.

The question remains open whether SSNS and SRNS are on the same spectrum, or distinct entities. It has become apparent that up to 30% of patients with SRNS could have Mendelian disease caused by genetic mutation.⁵ Since the advent of next generation sequencing (NGS), the discovery and extent of these gene mutations has grown rapidly, though exact incidence, particularly within the above clinical subgroups, remains unclear. Table 1 lists the 53 genes currently associated with SRNS.⁵⁻⁷

To date, studies on patients with NS have been limited by the number of genes covered, cohort number, and/or selection of phenotype included. We hypothesised that by applying clinically relevant inclusion criteria (Table 2) to a comprehensive cohort, utilising whole exome coverage to screen for all currently known causative genes, we could identify the true incidence and range of genetic disease in an otherwise unbiased population more accurately. Furthermore, we hypothesised that combining clinical criteria, including age of onset and steroid responsiveness, to comprehensive gene testing, would help to stratify between immunological/circulating factor disease and genetic disease, and better predict recurrence risk post-transplantation.

We screened the first 187 patients recruited to our national SRNS cohort, in an unbiased manner in order to understand the disease at the level of clinical presentation. The aim was to stratify for genetic vs 'circulating factor disease', and to determine whether this was linked to clinical predictability of disease progression, recurrence risk post-transplantation, and mechanistically targeted disease management.

Results

Cohort Characteristics and incidence of genetic disease

The clinical details of the 187 cases screened are presented in Table 3, with more detailed phenotyping shown in Table S2.

22/187 (12%) had familial disease, 164 were sporadic and 1 unknown (presumed sporadic). 59% (13/22) of patients in the familial group and 22% (36/164) in the sporadic group, had an identified mutation or a variant likely to be disease causing. The majority of familial patients

had a mutation in recessive, or dominant negative (*WT1*), genes (39 patients) and this group of patients had a trend towards an earlier age of onset compared to patients with a dominant gene mutation (10 patients) (Unpaired t test, two-tailed, P 0.07).

69/187 (36.9%) had developed end stage renal failure. 54 cases had undergone renal transplantation, the other 15 cases were on peritoneal dialysis or haemodialysis at last follow up. The subgroup that had reached chronic kidney disease (CKD) stage 5 and had a genetic aetiology of disease had an earlier age of onset compared to CKD stage 5 cases with SRNS in whom gene mutations had not been identified (4.75 vs 6.28 years, P value 0.0082, unpaired t test). No such difference was observed between patients with primary/presumed and secondary steroid resistance (4.86 vs 5.64 years, P value 0.6321, unpaired t test). Recurrence of the disease was noted in 27.8% of the transplanted patients. The rate of recurrence in those with a genetic form was 0/25 transplants, and with non-genetic/unknown was 15/29 transplants (51.7%) (Fisher's exact test, Two-sided, P<0.0001)

This phenotypic analysis confirms that patients with an identified gene mutation are more likely to progress faster to end stage renal failure (Figure 1). In this cohort 59.8% of genetic and 19.4% non-genetic/unknown SRNS patients reached CKD stage 5 within 4 years from diagnosis. The mean length of follow up for both patient groups was 7.6 years (min 1.00, max 18.03 years) and 6.2 years (min 0.58, max 16.55 years) respectively.

Patients with a mutation in *NPHS1* had an earlier mean age of onset than those with a mutation in *NPHS2*, 0.72 and 5.94 years respectively. 13 patients with congenital nephrotic syndrome (CNS) were found to have known or potential mutations in *NPHS1*, *LAMB2* and *WT1*.

Histopathological Findings

A renal biopsy was undertaken in 181/187 (96.8%) patients within 3 months of their date of diagnosis (3 patients with congenital nephrotic syndrome (CNS) were not biopsied and 3 patients' results were not available). The majority of patients had focal segmental glomerulosclerosis (FSGS) (54.1%→98/181) or minimal change (MCD) (23.8%→43/181) on biopsy however other variations (Table 3) were also reported. Biopsy reports did not correlate in any systematic manner with the genetic results identified.

Detailed Sequencing Results

Exome sequencing was performed on 187 paediatric SRNS/FSGS patients. Variants that fulfilled the filtering criteria to be causative (supplementary data) were detected in 17/53 known SRNS genes in 49/187 patients, 26.2% of the complete cohort, (not including *APOL1* risk G1 and G2 alleles), as shown in **Table 1**. A list of pathogenic variants is shown in Table 4. Of these, we identified 32 novel/rare variants that have not previously been reported in the literature. They are in the following genes: *NPHS1*, *NPHS2*, *ACTN4*, *TRPC6*, *MYO1E*, *COL4A3*, *DGKE*, *LMX1B*, *PODXL*, *OCRL*, *COL4A5*⁸, *ADCK4*⁹, *CRB2*¹⁰, *NUP93* and *NUP107*. The most frequently mutated genes in our cohort were *NPHS1*, *NPHS2* and *WT1* and this comprised 61.2% (30/49) of genetic detection in our cohort. Variants of unknown significance can be found in Table S3.

Analysis of genetic cohort

In total, 187 cases were sequenced, 96 male and 91 female, with a range of onset of NS between birth and 16 years (mean 5.6 years). No gender difference was observed between cases with a pathogenic gene variant identified and those without. Patients were grouped according to their age at onset of SRNS; the maximum prevalence occurred between 1-3 years old (51 cases) followed by 3-5 years old (34 cases). The highest proportion of genetic mutations was found in the 0-0.25 year group (13/15 or 87% of patients).

Variants Discovered in Commonly Associated Genes

In the < 2 years age group, *NPHS1* mutations were most frequently detected variants, namely in 14 patients with a mean age at onset of 0.72 years. Six novel likely disease-causing variants are described in Table 4. One of the sequenced patients (22) had congenital nephrotic syndrome (CNS) and a heterozygous mutation in *NPHS1* p.Arg1160*, previously described in patients with a similar phenotype.^{11,12} There were no other novel/rare variants in exons and splice site regions identified in *NPHS1* in this patient; however NGS analysis indicated the presence of a heterozygous deletion of exon 8. This deletion has subsequently been confirmed and investigated further by the use of bespoke custom designed MLPA probes. *WT1* mutations were the second most frequent, and detected in 4 patients with a mean age of onset of 1.68 years.

NPHS2 gene mutations were the most commonly detected in the > 2 years age group: 12 patients were found to have a known- or likely novel- disease causing variant with the mean age of onset of 5.94 years.

Variants Detected in genes less frequently presenting in childhood.

TRPC6 and *ACTN4* may cause both familial and sporadic juvenile and adult onset SRNS. It has been previously noted that mutations in these genes can sometimes cause disease with early childhood onset.^{5,13-15} Patients 30 and 38 were diagnosed at 7 and 3 years respectively, and found to have *TRPC6* mutations, and patient 29 was diagnosed at 12 years and had a novel *ACTN4* mutation. All 3 patients were sporadic.

We found several patients presenting overtly with SRNS, but associated with gene mutations usually linked to another renal phenotype. These were patient 33 with a *DGKE* mutation (usually atypical HUS), patient 44 with an *OCRL* mutation (usually Dent's disease type 2), and patient 32 with a *COL4A3* mutation (usually familial haematuria). Additional clinical details are presented within Supplemental data.

Other less common genes found mutated in this cohort included 2 patients (21 and 39) with *LMX1B* mutations (one with no extra-renal involvement), one novel variant in *PODXL* (patient 43), and 5 patients with the newly-described nucleoporin mutations, *NUP93* and *NUP107* (patients 45-48), making this a considerable subset of mutations within the cohort (10.2%).

Further clinical and genetic details of these patients are presented as Supplementary Data.

***APOL1* risk alleles**

Until recently *APOL1* risk alleles (for accelerated kidney disease) were described only in adult patients of African descent. Recently, Anyaegbu and colleagues showed that effects of *APOL1* nephropathy can also be found in young patients.¹⁶ 70% (130/187) of patients in our cohort are Caucasian whereas other ethnicities represented 30% (57/187). In our study, we found 3 patients (50, 95 and 101) of African descent with either homozygous or a compound heterozygous *APOL1* G1 and G2 risk variants. All 3 patients had presented before the age of 10 years and did not have any known or likely pathogenic variants in the nephrotic genes.

Discussion

This population-based exome sequencing approach has yielded several novel insights into SRNS. These include: the largest exome based study to date, and therefore the most accurate current incidence of monogenic disease, by taking into account all current genes implicated; An estimated incidence of circulating factor disease (CFD), based on clinical and genetic screening; The clinical utility of whole exome sequencing as the most relevant tool for screening in the rapidly evolving environment of monogenic SRNS; And a sufficiently sized population cohort from which to derive stratification based algorithms for clinical care

As clinicians treating children with nephrotic syndrome, one of the major barriers to optimal care is the inability to predict disease course and target treatment appropriately at an early stage after diagnosis. Stratifying patients by steroid sensitivity or resistance has been used effectively for many years. The recognition over the past decade of an increasing number of genetic causes for paediatric and adult SRNS provides an opportunity for further stratification and potential for personalised medicine. Using the data from the cohort presented here, which combines deep phenotyping with whole exome sequencing, we were able to explore how far we can stratify INS into genetic and circulating factor disease (CFD), with a potential proportion that can be placed outwith those groups.

The data suggest that there are two distinct categories of genetic and of circulating factor disease, with a significant proportion remaining 'unknown' almost exclusively with the clinical characteristics of primary steroid resistance and negative for genetic testing.

We have derived a clinically applicable working algorithm that firstly pinpoints the cohort likely to benefit from deep genetic analysis, the incidence of genetic disease in each subgroup of that cohort, and the broad range of genetic variation found. Further analyses include extracting recurrence risk post-transplantation and primary/secondary steroid resistance and correlating these clinical phenotypes with genotype.

In order to ascertain which patients to screen by whole exome sequencing, we performed an initial stratification step (Table 2), whereby patients with a specific secondary diagnosis by clinical criteria (e.g. obesity/hypertension related glomerulopathy, diabetic nephropathy) and/or biopsy evidence (e.g. membranous nephropathy, MPGN) would highly likely not have an underlying monogenic disorder related to the genes we have tested. It is possible that our clinical inclusion criteria excluded some patients who would be found to have a monogenic disease caused by one of the NS genes so far reported, but we estimated that this would be

unlikely. For example the NEPTUNE study included patients with diseases that we excluded, such as IgA nephropathy, membranous nephropathy and secondary FSGS, and found no patients in those subgroups with (SRNS) monogenic disease¹⁷.

We then used the clinical criteria of steroid resistance (primary, secondary or presumed in the congenital/familial cases) or idiopathic NS with FSGS on biopsy as the next level of stratification (Figure 3).

We found 26.2% (49/187) of the cohort has a single gene defect identified by screening the 53 currently known SRNS genes. By focusing on patients with no single gene defect identified, 13.8% of the cohort (25/181, Figure 3) have secondary steroid resistance and are highly likely to have CFD²⁶ (see below), and 59.7% (108/181) are in an 'unknown' category, with either an as yet undiscovered gene mutation, or CFD, or possibly a mechanism not yet suspected. This means that a maximum of 73.5% of patients could have CFD, although clearly more new genes are likely to be ascribed as pathogenic in the future to explain a proportion of this group. The risk in the 'genetic testing negative' subgroup of a 53.5% post-transplant recurrence risk could imply that CFD accounts for at least 53.5% of this group overall. However, this assumes no bias towards CFD in the group that has progressed to renal replacement.

We have previously shown that patients with secondary steroid resistance who reach established renal failure have a 90% chance of post-transplantation recurrence, the strongest indicator of circulating factor disease.¹⁸ This suggests that targeting selected patients earlier in their disease for intense immunosuppression may be beneficial, and a trial of for example pre-transplant plasma exchange in targeted patients could be designed.

The presumption that most genetic SRNS does not respond to immunosuppression is not proven, though potentially strengthened by this study. This needs further information from extended cohorts, as there are case reports of patients with a genetic mutation who have responded to steroids, at least partially (e.g. *PLCe1*, *EMP2*, *TRPC6*)^{19,20}. Since some genetic SRNS may respond, the question of whether response to secondary immunosuppression such as calcineurin inhibitors indicates a non-genetic disease remains open, and is a longer term aim of this cohort to address. Longitudinal correlation of proteinuria with medication history may be required to distinguish natural variations in glomerular protein leak as well as response to angiotensin converting enzyme inhibitors/receptor blockers (ACEi/ARBs).

We propose a robust filtering strategy (supplementary Methods) which has the ability to detect both known and novel variants according to clinically established criteria. We have highlighted the potential for detecting large deletions using exome sequencing data, which is confirmed by customised MLPA analysis, and should be considered at least in all patients in whom a single pathogenic variant (in a recessive gene) is found.

We propose that using a precision medicine approach of clinical phenotyping combined with deep sequencing, for patients with INS is a clinically actionable first step towards stratification into mechanistic groups in which response to immunosuppression, rate of progression to ESRF and risk of post-transplant recurrence can be quantified. This is of benefit to the refinement of focused clinical management and could be used to counsel patients/parents regarding prognosis. Furthermore, this level of stratification should be used to guide interventional clinical trial inclusion criteria, to target the most appropriate mechanistic patient groups.

Material and Methods

Between 2011 and 2015 we recruited patients with idiopathic nephrotic syndrome, with primary, secondary (defined as complete initial steroid response but during subsequent relapse no response to 4 weeks oral steroids) steroid resistance or familial NS. We excluded all those with a likely secondary glomerulonephritis either histologically (e.g. membranous nephropathy, IgA nephropathy, lupus nephritis), or clinically (e.g. diabetic nephropathy, obesity related glomerulopathy).⁶

Patients

Our cohort of paediatric SRNS cases was recruited via The United Kingdom Registry for Rare Kidney Diseases (RaDaR) and includes all tertiary paediatric nephrology centres in the UK. Detailed phenotypic information was entered online (www.renalradar.org), and laboratory data is automatically populated via links to the UK Renal Registry (www.renalreg.org). Appropriate informed consent from parents/carers is collected and assent from children for collection of data and genetic analysis obtained. The study was approved by the South West research ethics committee and institutional review board at each recruiting centre.

Inclusion criteria for paediatric patients to enter RaDaR (Table 2) included: Primary Steroid resistance, Congenital/Familial Nephrotic Syndrome (CNS) (presumed steroid resistance), secondary steroid resistance or FSGS on biopsy. Patients with syndromic proteinuric

nephropathy were also included. Patients with secondary NS (such as lupus nephritis or hypertension) were excluded. Recruited patients were entered onto RaDaR (www.renalradar.org) as described previously.⁶

Exome Sequencing

DNA from peripheral blood was extracted (Academic Renal Unit, Bristol) using either QIAamp DNA Blood Mini Kit or Gentra Puregene Blood Kit (Qiagen). Library preparation, sequencing and data generation was performed in the Genomics Core Facility of the Biomedical Research Centre at Guy's and St Thomas' Hospitals and King's College London. Mapping statistics are shown in Table S1, more detailed methods on variant calling are included in supplementary material.

Criteria for inclusion of variants as disease causing (full criteria are provided in supplementary methods)

Variants were included if: Minor allele frequency (MAF) below 0.01; variants not seen previously as homozygotes in any of the control databases; for autosomal dominant genes, variants not present in any control database; missense variants needed to be predicted by at least 2 in silico tools as potentially deleterious; synonymous and splice site variants were considered where there was a consistent predicted splice effect across the majority of tools; the amino acid must be conserved and not present in another multicellular organism.

Analytic Plan

We aimed to analyse the results of complete genetic testing in two ways. Firstly according to primary or secondary resistance (Figure 3), in order to address the hypothesis that secondary resistance is a surrogate for immunological disease and carries a high risk of post-transplant recurrence and low incidence of monogenic disease. Secondly according to a clinical pathway that would be of direct utility to physicians. Therefore we stratified patients into those with monogenic disease and those testing negative, then further separated according to primary or secondary steroid resistance. The categories are then presented according to clinical features pre-transplant, and risk of recurrence.

Disclosures/conflicts of interest

None.

Acknowledgments

We are indebted to the patients and families for participating in this study and the clinical teams involved in recruitment and sample collection.

This study was supported by Kids Kidney Research, Kidney Research UK, British Kidney Patient Association, North Bristol National Health Service Trust, and Nephrotic Syndrome Trust (NeST). The exome sequencing was supported by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Guy's and St Thomas' NHS Foundation Trust and King's College London. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health;

MN had a one year fellowship funded by the ERA-EDTA.

The UK Renal Rare Disease Registry (RaDaR) is funded by Kidney Research UK (KRUK) and British Kidney Patients Association (BKPA).

Author contributions:

MAS, AK, GIW conceived and designed the work. AB, HJM, KS, ESS, EC, WD, MN contributed to analysis, or interpretation of data for the work

LK, SH, DH, SM, SF, CJ, NW, MO, MC, RG, MS contributed to the acquisition of data, and helped with drafting and revision of the manuscript

Ethical approvals

The study was approved by the South West research ethics committee (reference 09/H0106/80) and institutional review board at each recruiting centre

Figure and table legends

Table 1. 53 Genes associated with steroid-resistant nephrotic syndrome (SRNS) of congenital, childhood, or adult onset.^{5-7,10,21-23} Genes with a likely or known mutation, or a risk allele, found in this cohort are highlighted in grey.

FSGS: Focal segmental glomerulosclerosis. CNS: congenital nephrotic syndrome. NS: nephrotic syndrome. SRNS; Steroid resistant nephrotic syndrome, SSNS – Steroid Sensitive Nephrotic Syndrome, SDNS – steroid dependent NS; DMS – diffuse mesangial sclerosis; AD – autosomal dominant, AR – autosomal recessive, DMS - Diffuse mesangial sclerosis, MPGN - Membranoproliferative glomerulonephritis, ESRD – End stage renal disease.

Table 2. Clinical criteria for inclusion

NS – nephrotic syndrome, FSGS – focal segmental glomerulosclerosis, SRNS – steroid resistant NS, SLE - systemic lupus erythematosus, HSP - Henoch-Schonlein purpura, MPGN - membranoproliferative glomerulonephritis, HIVAN - HIV-associated nephropathy.

Table 3. Summary of clinical details.

FSGS-Focal segmental glomerulosclerosis, MCD-Minimal Change Disease, MHc-Mesangial hypercellularity, DMS-Diffuse mesangial sclerosis, ESRF-End stage renal failure, GBM: glomerular basement membrane, *included because FSGS on biopsy or CNS

Table 4. Known and potential mutations in the 49 patients with monogenic nephrotic syndrome

(H) - homozygote, (Hemi) – hemizygote, F – familial, DP - disease associated polymorphism, SRNS - Steroid Resistant Nephrotic Syndrome, DMS - Diffuse mesangial sclerosis, aHUS - atypical Haemolytic uraemic syndrome, SIFT, MT and splicing predictions from Alamut, p – paternal, m – maternal, l - low risk, m - medium risk, NS - Nephrotic Syndrome, h - high risk, Y – deleterious, Na - not applicable, MT – MutationTaster, ExAC – The Exome Aggregation Consortium (<http://exac.broadinstitute.org/>), pos – This TRPC6 variant is considered possibly pathogenic here, a - patient included in²⁴, b - patient included in⁸, c - patient included in⁹, d - patient included in¹⁰, e – c.378+5G>²⁵, patients 26 and 27 are siblings, patients 45,46 and 47 are siblings. Using Alamut Visual 2.7 (SpliceSiteFinder-like, MaxEntScan, NNSPLICE, GeneSplicer, Human Splicing Finder) variants were considered likely pathogenic where there was a consistent predicted splice effect across the majority of tools.

Figure 1. Kaplan-Meier plot showing percentage of kidney survival in the genetic and genetic mutation negative group of patients.

Demonstrating faster rate of progression to End Stage Renal Failure (ESRF) in genetic vs. non-genetic cases. Log-rank (Mantel-Cox) Test (P value <0.0001).

Figure 2: Breakdown of monogenic causes vs Genetic testing negative patients, according to age of onset of disease

Figure 3. A precision medicine based guide to investigation of SRNS

* Patients 36, 88, 122, 160, 164, 187 are steroid sensitive (FSGS on biopsy) or unknown and thus are not included in the figure. KDIGO – Kidney Disease Improving Global Outcomes Guidelines. Age of onset of ESRF not known for patients 62 and 179 thus n=30 for CKD5 patients and n=28 for mean age of onset of ESRF; Mean age of onset of NS, and Mean age of onset of ESRF were compared and the only significant difference (#) was noted for Mean time to ESRF between primary+presumed monogenic and primary+presumed non-monogenic/unknown and secondary SRNS, with P value 0.0311 (two-tailed unpaired t test).

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